

AMENDMENTS TO THE SPECIFICATION

Please amend the following paragraphs in the specification as follows:

[0018] In other embodiments, the extracted fetal DNA may be labeled with a detectable agent or moiety before analysis by array-based comparative genomic hybridization. A detectable agent may comprise a fluorescent label. Suitable fluorescent labels for use in the practice of the methods of the invention may comprise fluorescent dyes such as, for example, ~~Cy 3TM, Cy 5TM, Texas red, FITC, Spectrum RedTM, Spectrum GreenTM~~ CYTM-3, CYTM-5, TEXAS REDTM, FITC, SPECTRUM REDTM, SPECTRUM GREENTM, phycoerythrin, a rhodamine, a fluorescein, a fluorescein isothiocyanine, a carbocyanine, a merocyanine, a styryl dye, an oxonol dye, a BODIPY dye, or equivalents, analogues, derivatives and combinations of these compounds. Alternatively, a detectable agent may comprise a hapten. Suitable haptens include, for example, biotin and dioxigenin.

[0047] In other embodiments, the first detectable agent and second detectable agents are ~~Cy 3TM and Cy 5TM, or Spectrum RedTM and Spectrum GreenTM~~ CYTM-3 and CYTM-5, or SPECTRUM REDTM and SPECTRUM GREENTM.

[0054] In certain embodiments, the nucleic acid segments of the test sample and reference sample are labeled with a detectable agent using such methods as random priming, nick translation, PCR or tailing with terminal transferase. In other embodiments, the first detectable agent and second detectable agents are ~~Cy 3TM and Cy 5TM, or Spectrum RedTM and Spectrum GreenTM~~ CYTM-3 and CYTM-5, or SPECTRUM REDTM and SPECTRUM GREENTM.

[0057] The inventive kits may optionally also contain materials to label a first sample of DNA with a first detectable agent and a second sample of DNA with a second detectable agent. Preferably, when the inventive kits comprise materials to label samples with detectable agents, the first and second detectable agents comprise fluorescent labels that produce a dual-color fluorescence upon excitation. For example, an inventive kit may contain materials to differentially label two samples of DNA with ~~Cy 3TM and Cy 5TM, or with Spectrum RedTM and Spectrum GreenTM~~ CYTM-3 and CYTM-5, or with SPECTRUM REDTM and SPECTRUM GREENTM.

[0061] **FIG. 1** presents a picture of an agarose gel (2% agarose/ethidium bromide stained), which shows that the samples of cell-free amniotic DNA labeled with ~~Cy 3TM~~ and the samples of

reference male DNA and reference female DNA labeled with ~~Cy-5TM~~ are uniformly amplified and labeled. Lanes 1 to 8 contain the four cell-free amniotic DNA samples (each sample was loaded twice in consecutive lanes). The controls are: ~~Cy-3TM~~, ~~Cy-5TM~~, CYTM-3, CYTM-5, reference male DNA and reference female DNA, which were loaded in lane 9, lane 10, lanes 11 to 15 and lanes 16 to 20, respectively. A molecular weight marker was loaded between lane 10 and lane 11.

[0097] The term “*differentially labeled*” is used to specify that two samples of nucleic acid segments are labeled with a first detectable agent and a second detectable agent that produce distinguishable signals. Detectable agents that produce distinguishable signals include matched pairs of fluorescent dyes. Matched pairs of fluorescent dyes are known in the art and include, for example, rhodamine and fluorescein, ~~Cy-3TM~~ and ~~Cy-5TM~~, and ~~Spectrum RedTM~~ and ~~Spectrum GreenTM~~, CYTM-3 and CYTM-5, and SPECTRUM REDTM and SPECTRUM GREENTM.

[0098] The terms “~~Cy-3TM~~” and “~~Cy-5TM~~”, “CYTM-3” and “CYTM-5” refer to fluorescent cyanine dyes (*i.e.*, 3- and 5-N,N'-diethyltetramethylindodicarbocyanine, respectively) produced by Amersham Pharmacia Biotech (Piscataway, NJ) (see, for example, U.S. Pat. Nos. 5,047,519; 5,151,507; 5,286,486; 5,714,386; and 6,027,709). These dyes are typically incorporated into nucleic acids in the form of 5'-amino-propargyl-2'-deoxycytidine 5'-triphosphate coupled to ~~Cy-3TM~~ or ~~Cy-5TM~~, CYTM-3 or CYTM-5.

[0099] The terms “~~Spectrum RedTM~~” and “~~Spectrum GreenTM~~”, “SPECTRUM REDTM” and “SPECTRUM GREENTM” refer to dyes commercially available from Vysis Inc. (Downers Grove, IL).

[0124] In certain preferred embodiments, amniotic fluid fetal DNA to be analyzed by hybridization is fluorescently labeled. Numerous known fluorescent labeling moieties of a wide variety of chemical structures and physical characteristics are suitable for use in the practice of this invention. Suitable fluorescent dyes include, but are not limited to: ~~Cy-3TM~~, ~~Cy-5TM~~, ~~Texas red~~, ~~FITC~~, ~~Spectrum RedTM~~, ~~Spectrum GreenTM~~, CYTM-3, CYTM-5, TEXAS REDTM, SPECTRUM REDTM, SPECTRUM GREENTM, phycoerythrin, rhodamine, fluorescein, fluorescein isothiocyanine, carbocyanine, merocyanine, styryl dye, oxonol dye, BODIPY dye (*i.e.*, boron dipyrromethene difluoride fluorophore), and equivalents, analogues or derivatives of these molecules. Similarly, methods and materials are known for linking or incorporating fluorescent dyes to biomolecules such as nucleic acids (see, for example, R.P. Haugland, “*Molecular Probes: Handbook of Fluorescent Probes and Research Chemicals 1992-1994*”, 5th Ed., 1994, Molecular Probes, Inc.). Fluorescent labeling agents as well as labeling kits are commercially available from, for example, Amersham Biosciences Inc. (Piscataway, NJ), Molecular Probes Inc. (Eugene, OR), and New England Biolabs Inc. (Beverly, MA).

[0125] Favorable properties of fluorescent labeling agents to be used in the practice of the invention include high molar absorption coefficient, high fluorescence quantum yield, and photostability. Preferred labeling fluorophores exhibit absorption and emission wavelengths in the visible (*i.e.*, between 400 and 750 nm) rather than in the ultraviolet range of the spectrum (*i.e.*, lower than 400 nm). Preferred fluorescent dyes include ~~Cy-3TM~~ and ~~Cy-5TM~~ CYTM-3 and CYTM-5 (*i.e.*, 3- and 5-N,N'-diethyltetramethylindo-dicarbocyanine, respectively). ~~Cy-3TM~~ 5TMCYTM-3 exhibits a maximum absorption at 550 nm; emits fluorescence with a maximum at 570 nm; and its fluorescence quantum yield has been determined to be 0.04 when ~~Cy-3TM~~ CYTM-3 is conjugated to a biomolecule (Amersham Biosciences Inc., Piscataway, NJ). ~~Cy-5TM~~ CYTM-5 displays absorption and emission fluorescent maxima at 649 and 670 nm, respectively, and its fluorescence quantum yield when conjugated to a biomolecule was reported to be 0.28 (Amersham Biosciences Inc., Piscataway, NJ). To increase the stability of ~~Cy-5TM~~ CYTM-5 (and therefore allow longer hybridization times as well as more intense fluorescence signals), antioxidants and free radical scavengers can be added to the hybridization mixture and/or to the hybridization/wash buffer solutions. ~~Cy-3TM~~ and ~~Cy-5TM~~ CYTM-3 and CYTM-5 also present the advantage of forming a matched pair of fluorescent labels that are compatible with most fluorescence detection systems for array-based instruments (see below). Another preferred matched pair of fluorescent dyes comprises ~~Spectrum RedTM~~ and ~~Spectrum GreenTM~~ SPECTRUM REDTM and SPECTRUM GREENTM.

[0168] The array used by the Applicants in a series of experiments described in Example 3 is the ~~GenoSensorTM~~ GENOSENSORTM Array 300 developed by Vysis. This genomic micro-array enables simultaneously screening for gene amplifications and deletions and provides a sensitivity that allows single gene copy detection. The Vysis arrays consists of 287 probe elements spotted in triplicate and comprises over 1300 gene loci derived primarily from bacterial artificial chromosomes (BACs), including microdeletion regions, important X/Y chromosome targets, aneusomy and aneuploidy of all chromosomes and telomeres.

[0186] Pairs of fluorescent labels are known in the art (see, for example, R.P. Haugland, "Molecular Probes: Handbook of Fluorescent Probes and Research Chemicals 1992-1994", 5th Ed., 1994, Molecular Probes, Inc.). Exemplary pairs of fluorescent dyes include, but are not limited to, rhodamine and fluorescein (see, for example, J. DeRisi *et al.*, Nature Gen. 1996, 14: 458-460); ~~Spectrum RedTM~~ and ~~Spectrum GreenTM~~ SPECTRUM REDTM and SPECTRUM GREENTM (commercially available from Vysis, Inc., (Downers Grove, IL)); and ~~Cy-3TM~~ and ~~Cy-5TM~~ CYTM-3 and CYTM-5 (commercially available from Amersham Life Sciences (Arlington Heights, IL)).

[0193] As described in Example 3, the system used by the Applicants is the micro-array technology system called ~~GenoSensor~~TM GENOSENSORTM that was developed by Vysis (see U.S. Pat. Nos. 5,830,645 and 6,159,685, each of which is incorporated herein by reference in its entirety). The ~~GenoSensor~~TM GENOSENSORTM Reader comprises a fluorescent imaging device with a Xenon-illumination source, an automated six-position filter wheel with three filters, a 1.3 million pixel high-resolution cooled CCD camera, an Apple Macintosh G4 computer with a 17" monitor. The ~~GenoSensor~~TM GENOSENSORTM software provide results of the karyotype analysis displayed as shown in Table 1 (Example 3).

[0241] The inventive kits may, additionally, contain materials to label a first sample of DNA with a first detectable agent and a second sample of DNA with a second detectable agent. Preferably, when the inventive kits comprise materials to label samples with detectable agents, the first detectable agent comprises a first fluorescent label and the second detectable agent comprises a second fluorescent label. Preferably, the first and second fluorescent labels produce a dual-color fluorescence upon excitation. For example, an inventive kit may contain materials to differentially label two samples of DNA with ~~Cy 3~~TM and ~~Cy 5~~TM, or with ~~Spectrum Red~~TM and ~~Spectrum Green~~TM CYTM-3 and CYTM-5, or with SPECTRUM REDTM and SPECTRUM GREENTM.

[0253] To determine if cell-free fetal DNA in amniotic fluid could be used for molecular karyotyping, cell-free DNA was extracted from eight frozen amniotic fluid supernatant samples from four known euploid males and four known euploid females. Each sample was ≥ 10 mL in volume and yielded between 200 and 900 ng of DNA. The samples were sent to Vysis for analysis. The results obtained by Vysis confirmed the quantity of DNA present. The concentration of DNA was adjusted to 25 ng/ μ L. Samples were labeled with ~~Cy 3~~TM and ~~Cy 5~~TM CYTM-3 and CYTM-5 according to the current labeling protocol for the ~~GenoSensor~~TM GENOSENSORTM Array 300. For each sample, reference male and female DNA of equal quantity was labeled for CGH. After DNase digestion, samples were visualized on a 2% agarose/ethidium bromide gel. As shown in Figure 1, DNA from samples and controls demonstrated uniform amplification and labeling.

[0259] Vysis has developed a novel microarray technology system that permits simultaneous assessment of multiple genomic targets. The ~~GenoSensor~~TM GENOSENSORTM system consists of the

following hardware: MacIntosh G3 PowerPC computer with 17" high resolution display monitor, 1.3 million pixel high-resolution cooled CCD camera, custom-designed optics, automated 6-position filter wheel with 3 filters, and xenon illumination source. The microarray consists of over 1,300 gene loci derived primarily from bacterial artificial chromosomes (BACs) as well as test and reference DNA that have been labeled with fluorophores. Using CGH, multiple clones of gene targets can be measured by analysis of fluorescent color ratios of the individual gene targets. The ~~GenoSensor~~TM GENOSENSORTM reader uses high resolution imaging technology to automatically acquire fluorescent images of the microarray within one minute. The reader software interprets the array image and determines copy number changes between the test and reference DNA.

[0262] In these experiments, all residual cells were removed from the amniotic fluid samples before DNA extraction. One hundred ng of each DNA sample was used per array. Test and reference samples were labeled with ~~Cy-3~~TM and ~~Cy-5~~TM CYTM-3 and CYTM-5, respectively and hybridized as described previously. Although hybridization was initially poor for all samples, adjusting the pH of the DNA samples to seven was found to increase the hybridization sensitivity and specificity. Two samples analyzed under these conditions were correctly identified as male, as the majority of X chromosome markers had significantly decreased hybridization compared to the reference female DNA and the *SRY* locus had significantly increased hybridization compared to the female reference, after normalization of the data. One of the two samples had been determined to originate from a fetus with trisomy 21 (karyotype 47, XY,+21, sample 02-1636). Analyzed by array-based comparative genomic hybridization, this sample was found to exhibit an increased hybridization on five of six chromosome 21 markers compared to the euploid reference DNA. However, the p-values were lower than 0.05 for only four of these markers and none of the p-values were lower than 0.005, which is the rigorous cutoff used by Vysis for these analyses.

[0267] Eight of the nine amniotic fluid cell-free DNA samples and all eight DNA samples from amniocytes led to correct identification of gender when hybridized to the Vysis ~~GenoSensor~~TM GENOSENSORTM microarray. One amniotic fluid cell-free sample (JH769) was not informative. Results obtained in both series of preliminary experiments are reported in the table of Figure 3 and in Figure 4. Overall, the data obtained shows that cell-free fetal DNA extracted from amniotic fluid supernatant can be a reliable source of nucleic acids for molecular karyotyping using microarrays.

[0272] When the hybridization performance of cell-free fetal DNA samples was compared with samples of DNA isolated from their corresponding amniocytes, the cell-free fetal DNA and cellular DNA samples were all informative for sex, but cell-free fetal DNA samples had higher clone-clone variability (noise). Noise in the samples was assessed using the median adjacent clone ratio difference (MACRD) criterion, calculated by determining the median of the absolute ~~Cy-3TM to Cy-5TM~~ CYTM-3 to CYTM-5 fluorescent intensity ratio difference between cytogenetically adjacent clones, which should be small. Currently, the “desirable” MACRD recommended by ~~GenoSensor~~ GENOSENSORTM analysis software for a high quality assay is < 0.065 (Vysis, unpublished data). Higher MACRDs indicate poor quality hybridization, since adjacent clone pairs have similar ratios in the vast majority of cases. On average, the MACRDs for DNA isolated from amniocytes were ≤ 0.065 , whereas cell-free fetal DNA samples exhibited values of 0.05-0.084. Although MACRDs were higher for some cell-free fetal DNA samples than for cellular DNA, in cell-free fetal DNA samples, the sensitivity of detection of chromosome-21, -X, and -Y markers, measured by normalized target/reference ratios of fluorescent intensities and *P* values, was similar, and quality values of array parameters, including mean intra-target coefficient of variation and modal distribution of standard deviation, were at or below the acceptable cutoffs established from multiple sets of hybridization done at Vysis for quality criteria development.